17 Haploid Cultures

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CHAPTER 17 CONCEPTS

- Haploids may be produced from male gametophytes (androgenesis) or female gametophytes (gynogenesis).

- *In vitro* androgenesis involves culture of anthers or isolated microspores.

- Gynogenesis may be induced in unfertilized ovule or ovary culture.

- Success of haploid cultures depends on genotype, media, and cultural conditions.

- Stage of microspore development at time of culture is critical to the success of androgenesis.

- Chromosome doubled haploids (diploids) are useful in breeding projects.

INTRODUCTION

The ability to produce haploid plants is a tremendous asset in genetic and plant breeding studies. Heritability studies are simplified, because with only one set of chromosomes, recessive mutations are easily identified. In addition, doubling the chromosome number of a haploid to produce a doubled haploid results in a completely homozygous plant. Theoretically, the genotypes present among a large group of doubled haploids derived from an F_1 hybrid represent in a fixed form the genotypes expected from an F_2 population. Use of doubled haploids in breeding programs can thus greatly reduce the time required for development of improved cultivars. To be most useful, a large number of haploids from many different genotypes are required.

Haploids have been available for genetic studies for many years. Prior to the 1960s, they were mostly obtained spontaneously following interspecific hybridization or through the use of irradiated pollen, but usually only infrequently and in very small numbers. Haploid methodology took a giant step forward 40 years ago when Guha and Maheshwari (1964) found that haploid plants could be obtained on a regular basis and in relatively large numbers by placing immature anthers of *Datura innoxia* Mill. into culture. This work was rapidly expanded using tobacco (*Nicotiana tabacum* L.), which became the model species for anther culture experiments. To date, androgenic haploids have been produced in over 170 species; several good reviews provide lists of these species (Maheshwari et al., 1982; Bajaj, 1983; Heberle-Bors, 1985; Dunwell, 1996). While efforts have been more limited, haploids have also been obtained from *in vitro* culture of the female gametophyte in over 30 species (Keller and Korzun, 1996; Lakshmi Sita, 1997). Gynogenesis has been successfully applied to several species in which androgenesis is generally ineffective, such as sugar beet (*Beta vulgaris* L.), onion *(Allium cepa* L.), and the Gerbera daisy (*Gerbera jamesonii* H. Bolus ex Hook).

Although much of the terminology used in this chapter has been discussed in previous chapters, the *in vitro* induction of haploids involves a few specialized terms. A haploid is a plant with the gametic or *n* number of chromosomes. Doubled haploids, or dihaploids, are chromosome doubled haploids or 2n plants. Androgenesis is the process by which haploid plants develop from the male gametophyte. When anthers are cultured intact, the procedure is called anther culture. Microspore culture involves isolating microspores from anthers before culture and is sometimes referred to as pollen culture. Haploids are derived from the female gametophyte through a process referred to as gynogenesis. *In vitro* gynogenesis involves the culture of unfertilized ovules or ovaries. While both androgenesis and gynogenesis may occur *in vivo*, the usage of the terms in this chapter will refer to the *in vitro* induction of haploids via these two mechanisms.

This chapter will begin with general discussions of androgenesis and gynogenesis, followed by a review of the factors that affect the successful production of androgenic and gynogenic haploids. Finally, some of the basic procedure used for the *in vitro* production of haploids will be summarized. Excellent discussions of *in vitro* haploid production, along with specific protocols for a number of crop species, can be found in Jain et al., 1996a; 1996b; 1996c; 1996d; 1997.

ANDROGENESIS

DEVELOPMENT OF HAPLOIDS

Haploid plants develop from anther culture either directly or indirectly through a callus phase. Direct androgenesis mimics zygotic embryogenesis; however, neither a suspensor nor an endosperm is present. At the globular stage of development, most of the embryos are released from the pollen cell wall (exine). They continue to develop, and after 4 to 8 weeks, the cotyledons unfold and plantlets emerge from the anthers. Direct androgenesis is primarily found among members of the tobacco (Solanaceae) and mustard (Cruciferae) families.

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During indirect androgenesis, the early cell division pattern is similar to that found in the zygotic embryogenic and direct androgenic pathways. After the globular stage, irregular and asynchronous divisions occur and callus is formed. This callus must then undergo organogenesis for haploid plants to be recovered. The cereals are among the species that undergo indirect androgenesis.

The early cell divisions that occur in cultured anthers have been studied (for review, see Reynolds, 1990). For species cultured during the uninucleate stage, the microspore either undergoes a normal mitosis and forms a vegetative and a generative nucleus or divides to form two similar-looking nuclei. In those cases where a vegetative and a generative nuclei are formed in culture, or where binucleate microspores are placed into culture, it is usually the vegetative nucleus that participates in androgenesis. The only species in which the generative nucleus has been found to be actively involved in androgenesis is black henbane (*Hyoscyamus niger* L.). When similar looking nuclei are formed, one or both nuclei may undergo further divisions. In some cases, the two nuclei will fuse, producing homozygous diploid plants or callus. Since diploid callus may also arise from somatic tissue associated with the anther, diploids produced from anther culture cannot be assumed to be homozygous. To verify that plants produced from anther culture are haploid, chromosome counts should be made from root tips or other meristematic somatic tissues (see Chapter 4). Because haploids derived from diploid species are expected to be sterile or have greatly reduced fertility, pollen staining, which is much quicker and requires less skill than chromosome counting, can also be used to identify and eliminate potential diploids. However, pollen staining may not distinguish between haploids and plants that have reduced fertility because they have a few extra or missing chromosomes (i.e., aneuploids). Haploids and diploids recovered from anther culture may also be distinguished by comparing size of cells, particularly stomatal guard cells, or through the use of flow cytometry.

PROBLEMS ASSOCIATED WITH ANTHER CULTURE

Problems encountered in plants during or as a result of anther culture range from low yields to genetic instability. Many of the major horticultural and agronomic crops do not yield sufficient haploids to allow them to be useful in breeding programs. In other species, genetic instability has often been observed from plants recovered from anther and microspore cultures.

The term "gametoclonal variation" has been coined to refer to the variation observed among plants regenerated from cultured gametic cells and has been observed in many species. While often negative in nature, some useful traits have been observed among plants recovered from anther and microspore culture. Gametoclonal variation may arise from changes in chromosome number (i.e., polyploidy or aneuploidy) or chromosome structure (e.g., duplications, deletions, translocations, inversions, and so on). In tobacco, gametoclonal variation has been associated with an increase in the amount of nuclear DNA without a concomitant increase in chromosome number (DNA amplification). In many cereals, a high percentage of the plants regenerated from anther culture are albino; changes in cytoplasmic DNA have been associated with this albinism. Good discussions and reviews of gametoclonal variation can be found in several chapters of Jain et al., 1996b.

TS: close space

GYNOGENESIS

As with androgenesis, gynogenic haploids may develop directly or indirectly via regeneration from callus (for review, see Keller and Korzun, 1996). The first cell divisions of gynogenesis are generally similar to those of zygotic embryogenesis. Direct gynogenesis usually involves the egg cell, synergids, or antipodals with organized cell divisions, leading first to the formation of proembryos and then to well-differentiated embryos. In indirect gynogenesis, callus may be formed directly from the egg cell, synergids, polar nuclei, or antipodals, or may develop from proembryos. Plants regenerated from callus may be haploid, diploid, or mixoploid. As with plants produced from anther cultures, chromosome counts can be used to identify haploids. Distinguishing between homozygous dihaploids, in which chromosome doubling occurred in culture, and diploids that developed from somatic tissue requires the use of molecular markers.

The major problems affecting the use of gynogenesis are the lack of established protocols for most species, poor yields, and production of diploid or mixoploid plants. Gametoclonal variation among gynogenic haploids has not been widely studied; however, it has been noted that, unlike androgenesis, gynogenesis of cereal species does not result in the production of albino plants.

FACTORS AFFECTING ANDROGENESIS

GENOTYPE

The choice of starting material for an anther or microspore culture project is of the utmost importance. In particular, genotype plays a major role in determining the success or failure of an experiment. Haploid plant production via androgenesis has been very limited or nonexistent in many plant species. Furthermore, within a species, differences exist in the ability to produce haploid plants. Even within an amenable species, such as tobacco, some genotypes produce haploids at a much higher rate than do others. Because of this genotypic effect, it is important to include as much genetic diversity as possible when developing protocols for producing haploid plants via anther or microspore culture.

CONDITION OF DONOR PLANTS

The age and physiological condition of donor plants often affect the outcome of androgenesis experiments. In most species, the best response usually comes from the first set of flowers produced

by a plant. As a general rule, anthers should be cultured from buds collected as early as possible during the course of flowering. Various environmental factors that the donor plants are exposed to may also affect haploid plant production. Light intensity, photoperiod, and temperature have been investigated, and at least for some species, these are found to influence the number of plants produced from anther cultures. Specific optimum growing conditions differ from species to species and are reviewed by Powell (1990). In general, the best results are obtained from healthy, vigorously growing plants.

STAGE OF MICROSPORE DEVELOPMENT

The most critical factor affecting haploid production from anther and microspore culture is the stage of microspore development; for many species, success is achieved only when anthers are collected during the uninucleate stage of pollen development. In contrast, optimum response is obtained in tobacco and *Brassica napus* L. from anthers cultured just before, during, and just after the first pollen mitosis (late uninucleate to early binucleate microspores).

In developing a protocol for anther culture, one anther from each bud is usually set aside and later cytologically observed to determine the stage of microspore development. In many cases, anthers within a bud are sufficiently synchronized to allow this one anther to represent the remaining cultured anthers. Measurements of physical characteristics of the flower, such as calyx and corolla length and anther color, shape, and size, are also recorded. Results of the experiments are analyzed to determine which microspore stage was the most responsive. The physical descriptions of the buds and anthers are then examined to determine if this microspore stage correlates to any easily identified inflorescence, flower or anther characteristic(s). For example, in tobacco, buds in which the calyx and corolla are almost identical in length usually contain anthers having microspores at or near the first pollen mitosis. A researcher wishing to produce a maximum number of haploid plants of tobacco would collect only buds fitting this physical description.

AU: "or flower"?

PRETREATMENT

For some species, a pretreatment following collection of buds, but before surface disinfestation and excision of anthers, has been found to be beneficial. Yields of tobacco haploids are often increased by storing excised buds at 7 to 8° C for 12 days prior to anther excision and culture (Sunderland and Roberts, 1979). For other species, temperatures from 4 to 10° C and durations from 3 days to 3 weeks have been utilized. For any one species, there may be more than one optimum temperature and length of treatment combination. In general, lower temperatures require shorter durations, whereas a longer pretreatment time is indicated for temperatures at the upper end of the cold pretreatment range mentioned above.

MEDIA

Androgenesis can be induced in tobacco and a few other species on a simple medium such as that developed by Nitsch and Nitsch (1969). For most other species, the commonly used media for anther culture include MS (Murashige and Skoog, 1962), N6 (Chu, 1978), or variations on these media. In some cases, complex organic compounds, such as potato extract, coconut milk, and casein hydrolysate, have been added to the media. For many species, 58 to 88 mM (2–3%) sucrose is added to the media, whereas other species, particularly the cereals, have responded better to higher (up to 435 mM or about 15%) concentrations of sucrose. The higher levels of sucrose may fulfill an osmotic rather than a nutritional requirement. Other sugars, such as ribose, maltose, and glucose, have been found to be superior to sucrose for some species.

For a few species, such as tobacco, it is not necessary to add plant growth regulators (PGRs) to the anther culture media. Most species, however, require a low concentration of some form of

auxin in the media. Cytokinin is sometimes used in combination with auxin, especially in species in which a callus phase is intermediate in the production of haploid plants.

Anther culture media is often solidified using agar. Because agar may contain compounds inhibitory to the androgenic process in some species, the use of alternative gelling agents has been investigated. Ficoll, Gelrite™ (Merck and Co., Inc., Rahway, NJ), agarose, and starch have proven superior to agar for solidifying anther culture media in various species (Calleberg and Johansson, 1996). The use of liquid medium has been advocated by some researchers as a way to avoid the potentially inhibitory substances in gelling agents. Anthers may be placed on the surface of the medium, forming a so-called "float culture." Alternatively, microspores may be isolated and cultured directly in liquid medium.

TEMPERATURE AND LIGHT

Various cultural conditions, such as temperature and light, may also affect androgenic response. Anther cultures are usually incubated at 24 to 25° C. In some species, an initial incubation at a higher or lower temperature has been beneficial. Haploid plant production was increased in *Brassica campestris* L. by culturing the anthers at 35° C for 1 to 3 days prior to culture at 25° C (Keller and Armstrong, 1979). In contrast, androgenesis was promoted in *Cyclamen persicum* Mill. by incubating cultured anthers at 5° C for the first 2 days of culture (Ishizaka and Uematsu, 1993).

Some species respond best when exposed to alternating periods of light and dark, whereas continuous light or dark cultural conditions have proven beneficial in other species. Other physical cultural factors, such as atmospheric conditions in the culture vessel, anther density, and anther orientation, have been studied and found to affect androgenic response in some species; however, species have varied greatly in their response to these physical factors.

FACTORS AFFECTING GYNOGENESIS

GENOTYPE

Gynogenesis has not been investigated as thoroughly or with as many species as has androgenesis; therefore, less information is available concerning the various factors that contribute to the successful production of haploids from the female than the male gametophyte. However, several studies have identified genotype as a critical factor in determining the success of an gynogenesis experiment. Not only are there differences between species, but genotypes within individual species have responded differently. As with androgenesis, it is important to include a wide range of genotypes in ovule and ovary culture experiments.

MEDIA

Media has also been identified as an important factor in gynogenesis. The most commonly used basal media for recovering gynogenic haploids are MS, B-5 (Gamborg et al., 1968), Miller's (Miller, 1963), or variations on these media. Sucrose levels have ranged from 58 mM to 348 mM (2–12%). While gynogenic haploids have developed in a few species without the use of growth regulators, most species have required auxins and/or cytokinins in the medium. For those species that undergo indirect gynogenesis, both an induction and a regeneration medium may be required. Most ovule and ovary culture experiments have been conducted using solid medium. A list of specific media components used for gynogenesis in several crop species can be found in Keller and Korzun (1996).

STAGE OF GAMETOPHYTIC DEVELOPMENT

Because the female gametophyte is difficult to handle and observe, determining the optimum stage of gametophytic development for gynogenesis is usually based on other, more easily discerned,

characteristics. Performance of ovule and ovary cultures has often been correlated with stage of microspore development. Depending on species, the best results have been obtained when the female gametophyte was cultured from the late uninucleate to trinucleate stage of megaspore development. In other studies, number of days until anthesis has been used as an indicator of stage of gametophytic development. A few gynogenesis studies that involved direct observations of the female gametophyte have been conducted. For several species, gynogenesis was most successful where cultures were initiated when the embryo sac was mature or almost mature (for review, see Keller and Korzun, 1996).

OTHER FACTORS

Cold pretreatment of flower buds at 4° C for 4 to 5 days has been effective in increasing yields of haploid embryos or callus in a few species, but has not been widely investigated. Seasonal effects have been observed in several species. Many of the other factors that affect androgenesis probably also affect gynogenesis; however, in most cases, insufficient data is available to detect trends in response. These variables should, however, be considered when initiating gynogenesis experiements.

GENERAL ANDROGENESIS PROCEDURES

COLLECTION, DISINFESTATION, EXCISION, AND CULTURE

Floral buds may be collected from plants grown in the field, greenhouse, or growth chamber. Entire inflorescences or individual buds are harvested and kept moist until ready for culturing. If buds are to be pretreated (i.e., kept at low temperature), they should be wrapped in a moistened paper tissue and placed into a small zipper-type plastic bag.

Flower buds are typically disinfested using a 5% sodium or calcium hypochlorite solution for 5 to 10 min, and then are rinsed thoroughly in sterile distilled water. Anthers are aseptically excised in a laminar flow hood, taking care not to cause injury. If the anther is still attached to the filament, the filament is carefully removed.

If a solid medium is used, the anthers are gently pressed onto the surface of the medium (just enough to adhere to the medium), but should not be deeply embedded. When using a liquid medium for intact anthers, the anthers are floated on the surface. Care must be taken when moving float cultures so as not to cause the anthers to sink below the surface.

For most species, disposable petri dishes are utilized for anther cultures. For a species with large anthers, such as tobacco, the anthers from 4 to 5 buds (20–25 anthers) may be cultured together on one 100 x 15 mm diameter petri dish. For species with smaller anthers, or for certain experimental designs, smaller petri dishes or other containers may be more useful. Petri dishes are usually sealed and placed into an incubator; the specific temperature and light requirements of the incubator depends on the species being cultured.

While many of the steps involved in microspore culture are similar to those of anther culture, microspore culture also requires the separation of the microspores from the surrounding anther tissue. Microspores may be squeezed out of anthers using a pestle or similar device, or a microblending procedure may be used. See Dunwell (1996) for a review of literature pertaining to microspore culture.

DETERMINING STAGE OF MICROSPORE DEVELOPMENT

For most species, stage of microspore development can be determined by "squashing" an entire anther in aceto-carmine or propiono-carmine and then observing the preparation under the low-power objective of a light microscope. The early uninucleate microspore is lightly staining with a centrally located nucleus. As the uninucleate microspore develops, its size increases and a large
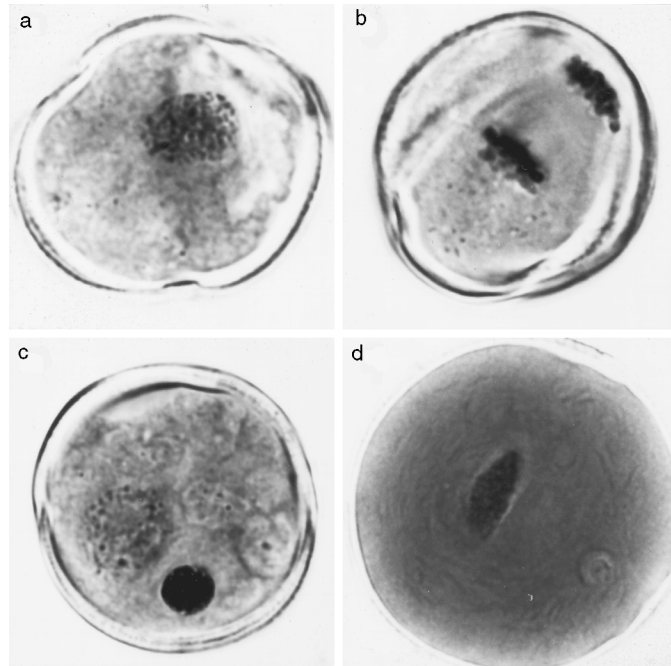
FIGURE 17.1 Microspores of tobacco. (A) Late uninucleate microspores. Vacuole can be seen on right side of cell. (B) Anaphase of first pollen mitosis. (C) Early binucleate microspore. Large diffuse vegetative nucleus and smaller, more darkly staining generative nucleus can be distinguished. (D) Mid-binucleate microspore. Staining is darker, obscuring vegetative nucleus, but the generative nucleus is still visible. (From Trigiano, R.N. and D.J. Gray, eds. 2000. *Plant Tissue Culture Concepts and Laboratory Exercises*, 2nd edition. CRC Press, Boca Raton, FL.)

central vacuole is formed. As the microspore nears the first pollen mitosis, the nucleus is pressed up near the periphery of the microspore (Figure 17.1A). Staining will still be fairly light. Pollen mitosis is of short duration, but it may sometimes be observed; it is recognized by the presence of condensed chromosomes (Figure 17.1B). The product of the first pollen mitosis is a binucleate microspore containing a large vegetative and a small generative nucleus. The vegetative nucleus is often difficult to recognize because it is so diffuse and lightly staining. However, this stage may be definitively identified by the presence of the small densely staining generative nucleus (Figure 17.1C). As the binucleate microspore ages, the intensity of the staining increases and starch granules begin to accumulate (Figure 17.1D). Eventually, both nuclei may be hidden by the dark staining starch granules.

HANDLING OF HAPLOID PLANTLETS

For species undergoing direct androgenesis, small plantlets can usually be seen emerging from the anthers 4 to 8 weeks after culture (Figure 17.2). When these get large enough to handle, they should be teased apart using fine-pointed forceps and then either placed on a rooting medium (usually low salt, with a small amount of auxin) or transplanted directly into a small pot filled with soilless potting mixture. The callus produced in species that undergo indirect androgenesis must be removed from the anther and placed onto a regeneration medium containing the appropriate ratio of cytokinin to auxin.

To produce dihaploid plants, it is necessary to double the chromosome number of the haploids, and for many species, a colchicine treatment is used. Published procedures for producing polyploids from diploids can be modified for use with anther culture derived haploids. For example, it may
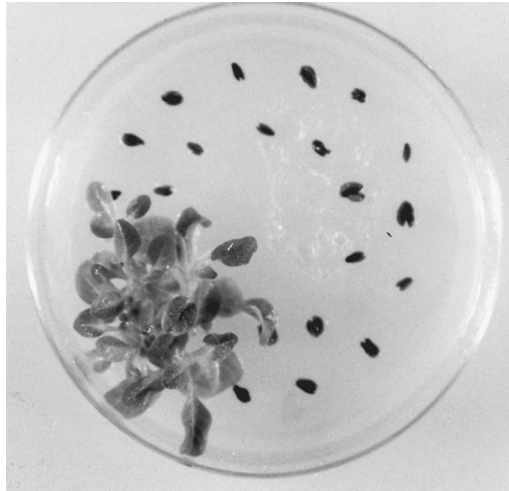
FIGURE 17.2 Haploid tobacco plantlets emerging from an anther. (From Trigiano, R.N. and D.J. Gray, eds. 2000. *Plant Tissue Culture Concepts and Laboratory Exercises*, 2nd edition. CRC Press, Boca Raton, FL.)

be possible to use a colchicine treatment designed for small seedlings with haploid plants directly out of anther culture. Alternatively, established procedures using larger plants may be used. In wheat and other cereals, chromosome doubling is induced by initially culturing anthers on a medium containing a low concentration of colchicine. In addition to leading to the direct regeneration of homozygous dihaploids, the inclusion of colchicine in the medium for the first few days of culture caused a decline in the number of albino regenerants (Barnabás et al., 2001).

GENERAL GYNOGENESIS PROCEDURES

Gynogenesis experiments are usually conducted using unfertilized ovules or ovaries, although entire immature flower buds have been cultured in a few species. It is easier to dissect ovaries than ovules without damaging the female gametophyte. However, in polyovulate ovaries, it may be advantageous to excise the ovules so that they can be in direct contact with the culture medium.

AU: "pollen is shed"?

Inflorescences must be collected before pollen shed, unless the species is highly self-incompatible or a male-sterile line is used. In developing a gynogenesis protocol for a species, it may be necessary to collect explants from several days before anthesis to just before anthesis. As discussed earlier, the stage of microspore development is sometimes recorded as an indicator of developmental stage of the female gametophyte. Procedures used for determining the stage of microspore development are described earlier in this chapter.

Disinfestation varies depending on species, growing conditions of explant source, and choice of explant. Woody plant material often requires longer disinfestation times and/or stronger sterilizing agents than herbaceous materials. Tissue from greenhouse-grown plants is usually easier to disinfest than that of field-grown plants. If ovules are to be cultured, a harsh surface sterilization procedure should be applied to ovaries. It should not be necessary to disinfest the ovules, since they are presumed to have been removed from a sterile environment inside the ovary. Commonly used sterilizing agents and disinfestation times are presented in Chapter 3 of this book.

Techniques used for the excision of ovules depend on the arrangement of ovules within the ovary. Care must be taken not to let ovules dry out during excision. A solid medium is typically used for gynogenesis experiments; choice of culture vessel depends on size of explant. Disposable petri dishes work well for culturing ovules of small-seeded polyovulate species, whereas test tubes may be preferable for large ovaries.

Handling procedures for gynogenic haploids are similar to those described for androgenic haploids. As plants emerge from cultured ovules or ovaries, they can be transferred to a rooting medium or transplanted directly to a soilless potting mixture. Colchicine or another mitotic inhibitor is typically used for doubling chromosome number to produce dihaploids.

SUMMARY

Haploids of many plant species can be produced *in vitro*. Anther culture has been the most widely used *in vitro* technique for producing haploids, but androgenic haploids have been obtained in a few species through the culture of isolated microspores. While fewer studies have been conducted involving the induction of haploids from the female gametophyte, gynogenesis has proven successful in several species. Yields of androgenic and gynogenic haploids differ greatly depending on species, and are also affected by cultural conditions, such as media formulation, stage of microspore or embryo sac at time of culture, and use of a low- or high-temperature pretreatment. Both androgenic and gynogenic haploids may arise directly, or may be produced indirectly through a callus intermediate. *In vitro*-derived dihaploids of several important crop species are now produced routinely. Use of dihaploids in breeding programs of these species has shortened cultivar development time. Expansion of this valuable breeding technique to additional species should occur as continued efforts are made to identify factors critical to *in vitro* induction of haploidy.

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AU: combine into one entry – "Vols. 1–5"?

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